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## *Pseudo-nitzschia* blooms, domoic acid, and related California sea lion strandings in Monterey Bay, California

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### ABSTRACT

Blooms of the toxin-producing diatom *Pseudo-nitzschia* commonly occur in Monterey Bay, California, resulting in sea lion mortality events. The links between strandings of California sea lions suffering from domoic acid (DA) toxicity, toxic cell numbers, and their associated DA concentration in Monterey Bay and in sea lion feces were examined from 2004 to 2007. While *Pseudo-nitzschia* toxic cells and DA concentrations were detectable in the water column most of the time, they were often at low levels. A total of 82 California sea lions were found stranded in the Bay between 2004 and 2007 with acute or chronic signs associated with DA

poisoning. The highest number with detectable DA in feces occurred in April 2007 and corresponded with the presence of a highly toxic bloom in the Bay. Higher DA levels occurred in feces from sea lions stranding with acute toxicosis and lower concentrations in feces of sea lions exhibiting signs of chronic DA poisoning or not exhibiting any neurologic signs. Results indicated that sea lions are likely exposed to varying levels of DA through their prey throughout the year, often at sublethal doses that may contribute to a continued increase in the development of chronic neurologic sequelae.

Key words: California sea lion, harmful algal blooms, domoic acid, *Pseudo-nitzschia*, acute toxicity, chronic neurologic, Monterey Bay, California.

Many marine mammal populations have been impacted significantly by fisheries and hunting by humans in past centuries. As a result, most of these populations were reduced to dramatically low numbers. Many such as the California sea lion, *Zalophus californianus*, recovered following implementation of federal protection in 1911 and 1972. California sea lions are now quite abundant, and although protected, are not threatened. Despite their high population numbers, several factors now affect sea lions off California, including environmental factors due to changes in oceanographic conditions such as El Niño that can cause prey fluctuations; bacterial diseases such as leptospirosis; and more recently the phycotoxins, that are now commonly present in the marine environment (Gulland and Hall 2007). Toxin producing harmful algal blooms (HABs) are increasing worldwide, and new events are documented regularly (Hallegraeff 1993, Van Dolah 2000, Anderson *et al.* 2002, Bates and Trainer 2006). These toxic algal blooms are also increasingly reported in areas where California sea lions feed, resulting in increased acute poisoning events and concern over the long-term effects of algal toxins on sea lion health (Lefebvre *et al.* 1999, Scholin *et al.* 2000, Gulland *et al.* 2002, Goldstein *et al.* 2008).

Some species within the diatom genus *Pseudo-nitzschia* have been shown to produce the neurotoxin domoic acid (DA), responsible for severe neurological and gastrointestinal illness in humans, also known as Amnesic Shellfish Poisoning (ASP) (Wright *et al.* 1989, Todd *et al.* 1993, Bates 2000). Most DA-producing *Pseudo-nitzschia* species are cosmopolites and common coastal phytoplankton (Hasle 2002). Over the past decade, blooms of the DA-producing diatom *Pseudo-nitzschia* have been the cause of numerous deaths of marine mammals and birds in California. The first major event that alerted the scientific community to the presence of this toxin occurred in 1991 in which more than 200 brown pelicans and Brandts cormorants were found dead on beaches in Monterey Bay, California (Fritz *et al.* 1992, Work *et al.* 1993). Since 1998, sea lion associated stranding events have been observed regularly off California (Lefebvre *et al.* 1999, Gulland 2000, Scholin *et al.* 2000, Gulland *et al.* 2002, Goldstein *et al.* 2008). *P. australis* was the primary DA source in these events, and filter-feeding anchovies (*Engraulis mordax*) were the apparent toxin vector (Fritz *et al.* 1992, Work *et al.* 1993, Walz *et al.* 1994, Lefebvre *et al.* 1999, Scholin *et al.* 2000). More recently, toxic *Pseudo-nitzschia* blooms have been increasingly observed in southern California coastal waters, with strandings of sea lions reaching or exceeding numbers found earlier in Monterey Bay (Schnetzler *et al.* 2007).

There is now sufficient evidence to suggest that coastal food webs are widely contaminated by DA during blooms of toxic diatoms, with sea lions being one of the most affected species that often come ashore when in distress, making them a

visible sentinel for a much more pervasive marine condition. The signs of acute DA poisoning in mammals include nausea, vomiting, diarrhea, and neurological signs including disorientation, seizures, coma, and death (Truelove and Iverson 1994). A short food chain is a key element for domoic acid intoxication, since it is a water-soluble toxin. Thus, unlike with lipid-soluble toxins, a vector of DA must either have cells and toxins in its gastrointestinal tract or have fed on a prey species containing toxin to act as a source to marine predators such as California sea lions.

The correlation with the presence of toxin producing cells, toxin in the water column and the number of sea lion strandings exhibiting signs of DA poisoning is not always clear. In the last 10 yr, at certain times in Monterey Bay, cell abundances, particulate DA concentrations, and sea lions stranding with neurological signs were not always well correlated; and conversely sea lions have stranded with neurological signs at times when domoic acid producing blooms were not evident along the California coast (Goldstein *et al.* 2008).

Recent work has shown that two separate clinical syndromes exist in sea lions: acute domoic acid toxicosis and a second novel neurological syndrome, chronic domoic acid toxicosis, characterized by epilepsy associated with chronic consequences of previous sublethal exposure to the toxin (Goldstein *et al.* 2008). While acute cases tend to strand in clusters, both spatially and temporally, chronic cases strand on beaches individually throughout the year at varying locations along the California coast. These chronically affected cases potentially explain some of the discrepancy between spatial and temporal changes in *Pseudo-nitzschia* cell abundance and particulate DA in the water column and sea lions stranding with neurological signs.

Here we examined potential links between strandings of sea lions suffering from both acute and chronic effects of toxicity, toxic cell numbers, and particulate DA in Monterey Bay and in feces from stranded sea lions from 2004 to 2007.

## MATERIALS AND METHODS

### *Water Sampling*

Surface water samples were collected at 13 different sites (Fig. 1) within Monterey Bay, California, from 2004 to 2007 where a monitoring program had been in place several years prior to the start of the present study to determine the spatial-temporal abundance of the toxin producing species of *Pseudo-nitzschia*, *P. australis*, and *P. multiseries* and their associated toxicity. Two sites, visited on a weekly basis during this period, were the Santa Cruz municipal wharf and MBARI station M1, ~20 km offshore over 1,000 m of water. Additional monthly surface water samples were collected from 11 other stations in the Bay through the "Wind to Whales" (W2W) project (NOAA). These latter surveys consisted of seven transect lines approximately 10 km in length from the 50 m isobath out to 122°05'W. Each hydrographic station included a phytoplankton net tow, a CTD cast, and a surface water sample for measuring cell abundance of toxic *Pseudo-nitzschia* and levels of particulate domoic acid.

### *Toxic Pseudo-nitzschia Cell Counts*

Cell counts were performed to estimate abundances of toxic *P. australis* and *P. multiseries* for each of the surface-water samples collected from all 13 sampling sites

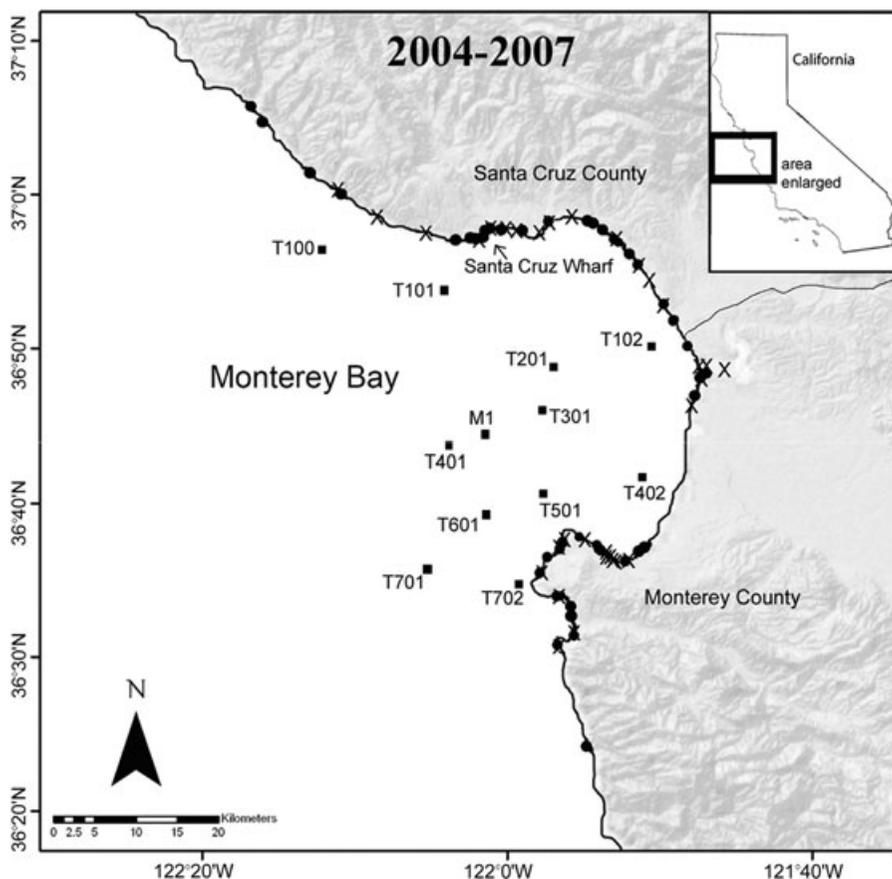


Figure 1. Map of Monterey Bay, California, showing sampling sites for toxic *Pseudo-nitzschia* cell counts and associated DA values, and sea lion stranding sites between 2004 and 2007. Dark circles (●) represent acute stranding cases and crosses (x) represent chronic stranding cases. Note that “T” stations were visited monthly on the W2W cruises, and the nearshore Santa Cruz municipal wharf and open-water MI stations were visited weekly during the study period.

(Santa Cruz + MBARI + 11 W2W sites, Fig. 1) over a 4 yr time course. Whole cell hybridization with species-specific large subunit (LSU) rRNA-targeted fluorescent probes (Miller and Scholin 1996) were used for each of these collected samples to distinguish the toxic species from the other, nontoxic species of *Pseudo-nitzschia* when making cell counts, and to enumerate the DA-producing cells. Five to 30 mL aliquots of seawater were filtered onto 1.2  $\mu\text{m}$  isopore polycarbonate filters (Millipore) and preserved with a saline ethanol solution for at least 1 h. After rinsing with hybridization buffer (5X SET [ $1/5$  dilution of 3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl, pH 7.8], 0.1% [v/v] IGEPAL-CA630 [Sigma, St. Louis, MO], 25 mg/mL polyadenylic acid [Sigma]), each sample was incubated with species-specific probes for *P. australis*, *P. multiseriis*, a positive eukaryote control probe, and a negative control probe designed for *Alexandrium tamarense*. A third control consisted of a sample with no probe added. After 1 h, filters were rinsed and

placed on microscope slides. Intact cells that retained the fluorescein labeled probe were then counted on a Zeiss Standard 18 compound microscope, equipped with epifluorescence (microscope illuminator 100, Zeiss Inc., with high pressure mercury lamp, 50 W). The entire area of each filter was counted and used to generate results that were converted into cells per liter.

#### *Domoic Acid Detection in Water Samples*

Domoic acid (DA) concentrations in particulate matter (*i.e.*, cells) were determined using the FMOC-HPLC method described by Pocklington *et al.* (1990). Pre-column derivatization of DA in cell extracts with 9-fluorenylmethylchloroformate (FMOC) reagent were used to form the fluorescent FMOC-derivative of DA. Prior to analysis, particulate samples collected on GF/F filters (Whatman Inc.) were extracted in 2.5 mL 10% aqueous methanol. Filters were vortexed to homogenize cells, followed by sonication using a sonicator probe for 2 min. Extracts were centrifuged for 3 min at 1399 × g, the supernatant removed, and filtered through a 0.22 μm filter (Millipore Corp., Bedford, MA). For derivatization, 200 μL of extract was vortexed with 50 μL 1 M borate buffer (pH 6.2), 10 μL dihydrokainic acid (DHKA) internal standard (100 μg/mL DHKA in 10% aqueous methanol) for 10 s, and then 250 μL FMOC-Cl reagent was added and vortexed again for 45 s. Three ethyl acetate washes were performed and the aqueous layer was removed for HPLC analysis. A 5 μL sample was injected into a Hewlett-Packard HP1090M HPLC equipped with an HP1046A fluorescence detector set for excitation at 264 nm and emission at 313 nm with a mobile phase flow rate of 0.2 mL/min comprised of 40% aqueous acetonitrile, 0.1% TFA. Isocratic separations were performed on a reverse phase C<sub>18</sub> column (2.1 mm × 25 mm, Vydac 201TP52; Separations Group) heated to 40°C. A calibration curve was generated using the DACS-1C standard (Canadian National Research Council, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS, Canada) of 5, 10, 25, 50, 100, and 250 ng/mL in 10% aqueous methanol ( $r^2 = 0.99$ ).

#### *Stranded Sea Lions*

Sea lions found ill along the Monterey Bay coast from 2004 to 2007 were transported to The Marine Mammal Center in Sausalito, California (TMMC) for clinical examination, treatment, and rehabilitation for release back to the wild, when possible. Stranding data collected included sex, age class, stranding date and location, and cause of stranding. Sex determination was based on genital morphology. Animals were categorized by age class according to growth curves based on sex, body length, weight, development of teeth, and color of skin or pelage, and other external characteristics (Greig *et al.* 2005): pup (0–1 yr), yearling (1–2 yr), juvenile male (2–4 yr), subadult male (4–8 yr), subadult female (2–5 yr), adult male (8+ yr), and adult female (5+ yr). Strandings were classified as acute or chronic domoic acid toxicosis cases as defined in Goldstein *et al.* (2008) or “other” if they did not exhibit any of the neurological signs described above, showed clinical signs associated with other disease processes, or died due to other causes.

#### *Domoic Acid Detection in Sea Lion Feces*

DA was measured in a subset of stranded sea lions ( $n = 58$ ) for which feces was available between 2004 and 2007. Feces from 35 stranded individuals that exhibited

(1) acute ( $n = 8$ ), (2) chronic ( $n = 3$ ) signs of DA poisoning, or (3) did not exhibit neurologic signs and stranded due to other causes ( $n = 24$ ) were collected within 24 h of admission; and feces from 23 stranded individuals that exhibited acute ( $n = 6$ ), chronic ( $n = 5$ ) signs of DA poisoning, or did not exhibit neurologic signs and stranded due to other causes ( $n = 12$ ) were collected after 24 h of admission. DA extractions were performed as previously described by Lefebvre *et al.* (2001). A 1:4 ratio of sea lion feces (wet weight) to 50% aqueous methanol extraction solvent was vortexed for 30 s, homogenized for 2 min with a homogenizer probe on ice, sonicated for 2 min (30–40 W) in an ice bath with a Sonicator 3000 equipped with a microtip (Misonix), and then centrifuged for 20 min ( $1399 \times g$ ). The supernatant was then collected and passed through a 0.22  $\mu\text{m}$  syringe filter (Millipore Corp., Bedford, MA). Consequently, 2–4 mL of filtrate were passed through a strong anion exchange (SAX) solid phase extraction (SPE) column (JT Baker) which was preconditioned with 6 mL nanopure water, followed by 3 mL 100% aqueous methanol, and finally 3 mL 50% aqueous methanol. After washing the column with 5 mL of 10% acetonitrile, DA was eluted with 5 mL of 0.5 M NaCl in 10% acetonitrile at a rate of one drop per second (Hatfield *et al.* 1994). The column was not allowed to run dry at any time during solid phase extraction.

Sea lion fecal samples were analyzed for the presence of DA using an isocratic elution profile on a Hewlett-Packard 1090 HPLC equipped with a diode array detector (DAD) set at 242 nm with a bandwidth of 10 nm. The reference signal was set at 450 nm with a bandwidth of 10 nm. A reverse phase Vydac C<sub>18</sub> column (catalog #201TP52, 2.1 mm  $\times$  25 mm, Separations Group, Hesperia, CA) equipped with a Vydac guard column (particle size 5  $\mu\text{m}$ ) was used. The mobile phase (90/10/0.1, water/acetonitrile/TFA) was degassed with helium for 10 min prior to analysis. A calibration curve was generated using DACS-1C DA standards of 0.15, 0.3, 0.5, 1.0, 2.0, 4.0, 8.0, and 16  $\mu\text{g}/\text{mL}$  ( $r = 0.99$ ). The instrument detection limit, which is equivalent to the concentration that corresponded to three times the standard deviation of the signal from the lowest detectable standard ( $n = 3$ ), was 0.018 ppm. Injections were 20  $\mu\text{L}$  with a flow rate of 0.3 mL/min. Two DA-free sea lion fecal samples were selected and spiked with 16 ppm DA (DACS-1C) to test the matrix interference for the instrument. Spiking recoveries were 94.8% and 101.8%, respectively.

#### *Electron Microscopy for Pseudo-nitzschia Species Identification in Sea Lion Feces*

Transmission electron microscopy (TEM) was used to determine the presence of *Pseudo-nitzschia* in sea lion feces using methods slightly modified from Miller and Scholin (1998). Sea lion feces were concentrated onto 1.2  $\mu\text{m}$  pore size isopore polycarbonate membrane filters (Millipore). Salt was removed from samples by rinsing with deionized water under low vacuum pressure (150 mmHg). Organic material was removed with saturated  $\text{KMnO}_4$  added until the filters were covered and allowed to digest the samples for 45 min, followed by the addition of 3 mL HCl to the water samples until the color became clear. Samples were held for at least 60 min in 3 mL of HCl to complete the oxidation process. Samples were then vacuumed gently and rinsed with deionized water. This process was repeated twice. After cleaning, the filter was submerged in 0.5 mL of MilliQ water in a microcentrifuge tube to resuspend diatom frustules in solution. The water was affixed onto a 100 mesh copper grid with a formvar carbon support film. Finally, the samples were placed in a dessicator to dry before viewing them on a Jeol 100CX TEM.

### Data Analysis

The time series data for toxic *Pseudo-nitzschia* (PN), DA, acute, and chronic stranded sea lions were averaged monthly for 41 mo. For time series data, a standard analysis to quantify the variations at different frequencies is the power density (PD) spectrum analysis (e.g., Emery and Thomson 2004). Because more and more biological data are time series, it is important that standard techniques are applied for their analysis. PD analysis is a variation of the Fourier analysis. Fourier analysis is very similar to calculations of correlation coefficient over a range of frequencies. It captures the variability at various frequencies. This “various frequencies” is determined by the actual process, but the characteristics are determined by (1) time interval of the data, and (2) total length of the record. The former determines the highest frequency (the Nyquist frequency) that can be resolved, the latter determines the frequency resolution (how fine the peaks can be to separate different frequencies from each other): i.e.,  $df = 1/T$ , in which  $df$  is the frequency resolution of the PD analysis (or how close two nearby peaks in frequency domain can be before they appear to be merged into one signal peak),  $T$  is the total length of the record. In our study, in order to better quantify the annual variations in reference to variabilities at other periodicities (or frequencies), such a PD spectrum analysis was performed on the time series data (Fig. 2). For better visualization of the data, the time series data was zero-padded (Emery and Thomson 2004), a standard technique for presentation without introducing any artificial information. The zero-padding was done by adding zeros five times the original length of the data at the end of the data before the PD analysis. The fast Fourier transform (FFT) was then applied to obtain the PD spectrum of each time series. Note: “zero-padding” only improves the quality of the peaks visually, but not the nature of the spectrum (e.g., the number of peaks and the location and magnitude of the peaks at different frequencies). The power density spectrum analysis provides a clear presentation of the major frequencies and relative magnitude of variations of the data.

In addition to the power density spectrum analysis, the log linear correlation of the data was also examined. The analysis was carried out as a log linear regression between PN and DA, DA and acute strandings, and PN and acute strandings. Log linear regressions among acute strandings, PN, and DA (acute strandings expressed as linear combination of PN and DA, all in log scale); and chronic strandings, PN, and DA (chronic strandings expressed as linear combination of PN and DA, all in log scale) were also performed. Finally, a log linear regression among acute strandings, PN, DA, and chronic strandings (acute strandings expressed as linear combination of PN, DA, and chronic strandings, all in log scale) was performed to quantify the correlation of the obtained parameters. In mathematical format the regression analyses used the following equations:

$$\log_{10} DA = a \log_{10} PN + b \quad (1)$$

$$\log_{10} AC = a \log_{10} DA + b \quad (2)$$

$$\log_{10} AC = a \log_{10} PN + b \quad (3)$$

$$\log_{10} AC = a_1 \log_{10} PN + a_2 \log_{10} DA + b \quad (4)$$

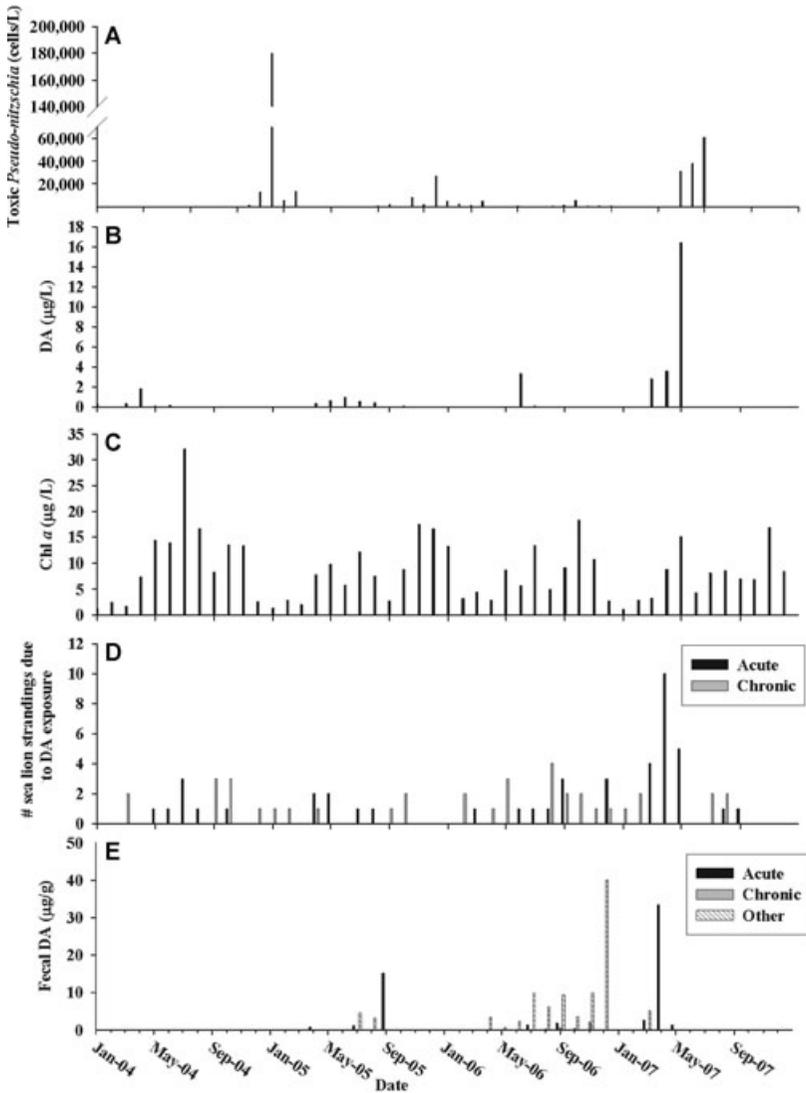


Figure 2. Time-series data, from 2004 to 2007 in Monterey Bay, California: (A) the abundance of toxic *Pseudo-nitzschia* cells (*P. australis* + *P. multiseries*), monthly average cells/L for all the stations sampled in the Bay, (B) particulate DA levels (monthly average  $\mu\text{g/L}$  for all the stations sampled in the Bay), (C) phytoplankton biomass (Chl *a*  $\mu\text{g/L}$  for Santa Cruz site only), (D) strandings of California sea lions, *Zalophus californianus*, with acute and chronic signs of DA toxicity (monthly total for the entire Bay), and (E) domoic acid (monthly average  $\mu\text{g/g}$ ) in sea lion feces (acute + chronic + others) collected during the study interval.

$$\log_{10} CN = a_1 \log_{10} PN + a_2 \log_{10} DA + b \quad (5)$$

$$\log_{10} AC = a_1 \log_{10} PN + a_2 \log_{10} DA + a_3 \log_{10} CN + b \quad (6)$$

in which  $a$ ,  $b$ ,  $a_1$ ,  $a_2$ , and  $a_3$  are coefficients being determined by the regression analysis in a least-squared sense. DA and PN represent the concentrations of domoic acid and *Pseudo-nitzschia*, respectively, while AC and CN represent acute strandings and chronic strandings, respectively.

## RESULTS

From 2004 to 2007 the Monterey Bay area (Fig. 1) appeared to have anomalously small and infrequent events of toxic *Pseudo-nitzschia* (*P. australis* and *P. multiseriata*) blooms producing the toxin domoic acid (Fig. 2A, 2B). During this period the dominant *Pseudo-nitzschia* species was *P. australis*, a species previously associated with acute toxicity and pinniped mortalities.

These data indicated that toxic *Pseudo-nitzschia* cells were detectable in the water most of the time, but cell abundance was low (cell abundance was  $<10^3$ /L 60% of the time). A seasonal pattern was observed in the Bay stations with peak toxic cell numbers ranging  $>10^3$ /L occurring each spring throughout the study (Fig. 2A). The spectrum analysis showed clearly that there was an annual cycle for toxic *Pseudo-nitzschia* (peak at 1 cycle per year, or CPY, in Fig. 3A). Highest toxic cell numbers (up to  $9 \times 10^5$  cells/L) were seen in April 2004 from the Santa Cruz municipal wharf followed by spring of 2007 (March–May) from both Santa Cruz municipal wharf and M1, offshore station (up to  $4 \times 10^5$  cells/L) (station specific data is not shown) (Fig. 2A). Chl  $a$  values, from the Santa Cruz site, ranged from 0.5 to 110.6  $\mu\text{g/L}$  (Fig. 2C) in the Bay. On many occasions, highest Chl  $a$  values occurred when toxic *Pseudo-nitzschia* presence in the phytoplankton community was low or nondetectable, demonstrating no significant correlation between the two ( $r^2 = 0.08$ ).

DA concentrations measured in water aliquots also used for toxic *Pseudo-nitzschia* cell counts showed that they were significantly correlated with cell abundance of the two toxic *Pseudo-nitzschia* species with similar annual cycle ( $r^2 = 0.50$ ) (Fig. 2B, Fig. 3B, Fig. 4A). The highest average DA concentrations for the Bay were measured between March and May 2007, reaching 16.4  $\mu\text{g DA/L}$ , and correlated with high levels ( $>10^4$ ) of *Pseudo-nitzschia* cells/L present in the water that resulted in DA cell quotas of  $30.3 \pm 17.6$  pg DA/cell.

A total of 82 California sea lions stranded in Monterey Bay from 2004 to 2007 with signs associated with acute or chronic domoic acid poisoning: 44 acute cases and 38 chronic neurologic (Fig. 2D, Table 1). The number of sea lion strandings was compared to the timing of toxic *Pseudo-nitzschia* and DA presence in Monterey Bay over the same time period (Fig. 2A, B, D, Fig. 4B, C). Overall, it appeared that there was a poor correlation between sea lions stranding with DA-related neurologic signs (acute and chronic), the presence of toxic *Pseudo-nitzschia*, and domoic acid concentration in the bay ( $r^2 = 0.20$ ). When comparing the timing of stranding for the sea lions with signs of acute toxicosis with cell numbers of the DA producers and associated DA concentrations detectable at the Bay stations, a similar annual cycle was observed (Fig. 3C) and a better correlation was found between DA presence ( $r^2 = 0.34$ ), but not with *Pseudo-nitzschia* cell numbers alone ( $r^2 = 0.04$ ) (Fig. 4B, C). Chronic neurologic cases were observed throughout the year with no annual cycle (Fig. 3D) and often when DA producer cell numbers and associated DA concentrations were low or nondetectable ( $r^2 = 0.01$  and  $r^2 = 0.04$ , respectively). Even though there were variable peaks at frequencies below or above 1 CPY, the 1 CPY peak was larger than its nearby peaks for toxic *Pseudo-nitzschia*, DA, and acute stranding

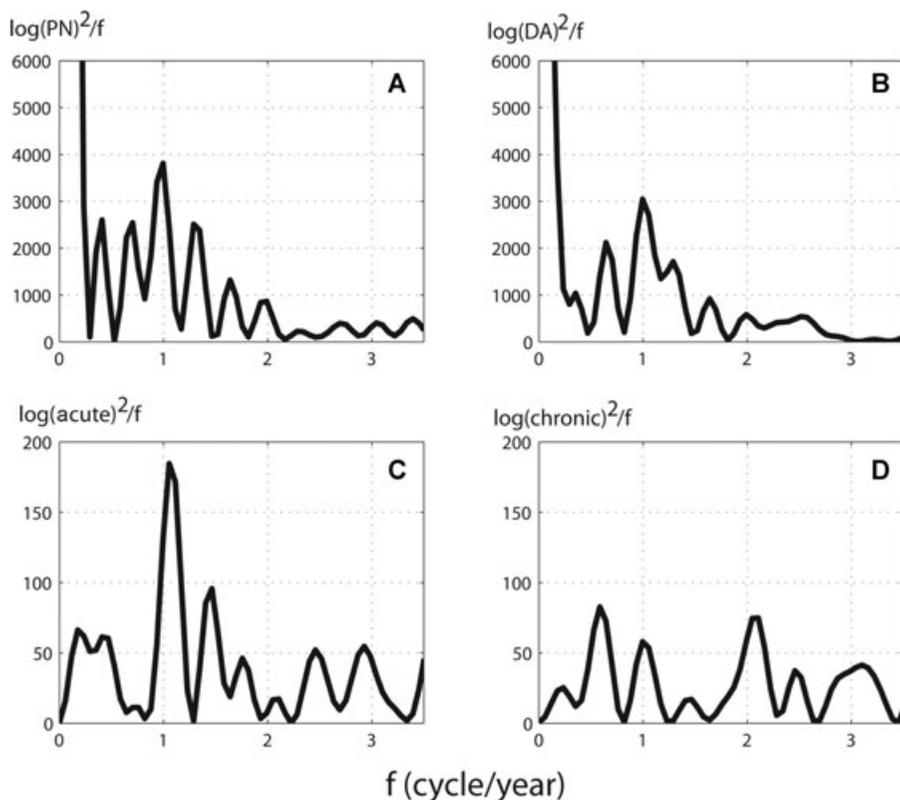


Figure 3. Power density spectrum (PS) analysis showing the distribution of abundance/concentration variability at various frequencies. Despite the multifrequency nature, the data showed a frequency at about 1 cycle per year (CPY), indicating the annual cycle for (A) toxic *Pseudo-nitzschia*, (B) domoic acid concentrations and (C) acute stranding data, but not for (D) the chronic stranding data.

data, but not for chronic stranding data. This seems to be because the acute cases correlate better with the blooms of *Pseudo-nitzschia*. The chronic cases may have more noise, with many other undefined factors that are not considered. The peak at annual cycle for the chronic data is of lower magnitude. The highest numbers of sea lions stranding with acute DA toxicity were seen in spring (March–May) of 2007, when a highly toxic bloom of *Pseudo-nitzschia* was also observed in the Bay (Fig. 2A, D). The highest number of strandings of chronic cases occurred throughout 2006 when toxic *Pseudo-nitzschia* numbers were moderate to low in the Bay (Fig. 2A, 2D). Table 2 summarizes the regression results using Equations 1–6 as shown in Figure 4. All the estimated coefficients obtained from the regression analysis are shown in Table 2. In addition, standard deviation (SD), confidence interval for the regression analysis (CI), and  $r^2$  values are included.

Sea lions stranded with neurological signs at many different sites in Monterey Bay between 2004 and 2007 (Fig. 1). The chronic neurologic cases stranded at varying locations throughout the Bay, which was most apparent in 2006 when we observed

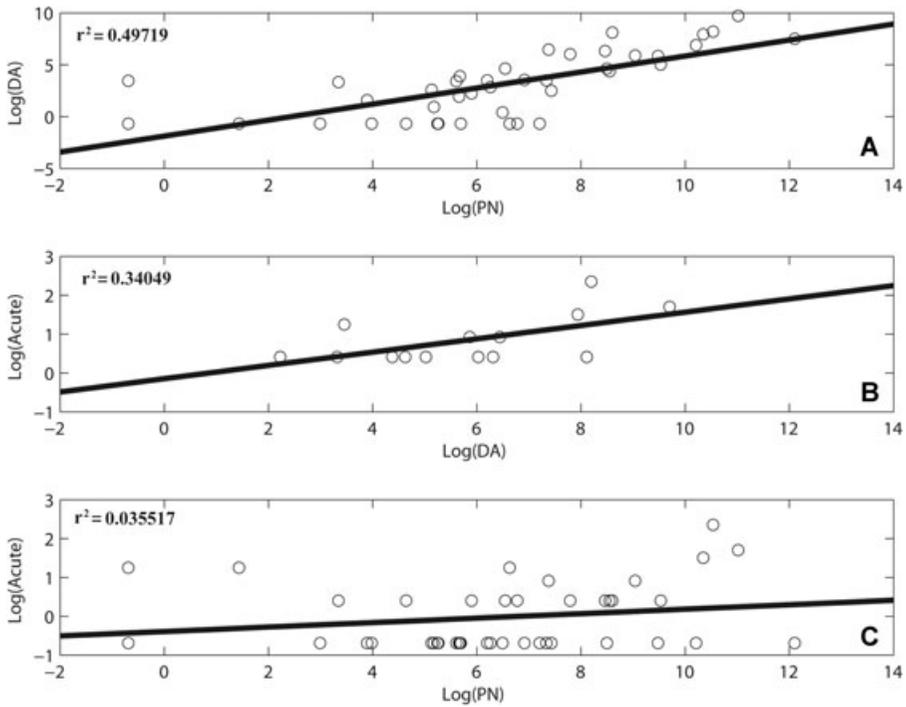


Figure 4. Linear regression (log scale) showing the relationship between (A) *Pseudo-nitzschia* (PN), and domoic acid (DA), (B) DA and acute stranding, and (C) PN and acute stranding.

the highest number of chronic cases. The acute neurologic cases, on the other hand, stranded in clusters along the Bay at times when high cell numbers and DA was present in water samples, which was most apparent in April 2007. Chronic neurologic cases that stranded in 2006 consisted mainly of subadult females and males, while the acute cases stranding in 2007 consisted mainly of adult and subadult males (Table 1). Overall, of the 44 acute cases, the majority was subadult and adult males, and the 38 chronic cases were mostly adult female or juvenile males (Table 1).

Feces, collected between 2005 and 2007, from 58 stranded individuals that (1) exhibited acute ( $n = 14$ ), (2) chronic ( $n = 8$ ) signs of DA poisoning, or (3) did not exhibit neurologic signs and stranded due to other causes ( $n = 36$ ) (Table 1) were analyzed for the presence of DA. Most of the samples from varying concentrations of DA. The highest DA concentration was found in feces collected from stranded individual that exhibited acute signs of DA poisoning in April 2007 ( $96.8 \mu\text{g DA/g}$ ), also corresponding to the highly toxic bloom and highest number of sea lions stranding with acute toxicity in the Bay (Fig. 2A, D, E). Most of the sea lions exhibiting acute signs of DA poisoning (71%) contained detectable but highly varying levels of DA concentrations in feces (ranging from 0.2 to  $96.8 \mu\text{g DA/g}$ , with mean value of  $9.1 \pm 25.5 \mu\text{g DA/g}$ ). The presence of *Pseudo-nitzschia* frustules was confirmed by TEM in selected samples from sea lions with acute toxicity and *P. australis* was found to be the dominant species. This finding was consistent with those from the water column samples, but *P. multiseriata*, *P. pseudodelicatissima* and

*Table 1.* California sea lions stranding in Monterey Bay between 2004 and 2007 with acute and chronic domoic acid toxicity. Parentheses in italics are the number of stranded California sea lions with feces examined for the presence of DA. Animals stranded due to other causes were categorized as "Other" and all had feces examined for the presence of DA. Note: age classes were defined as: pup (0–1 yr), yearling (1–2 yr), juvenile male (2–4 yr), subadult male (4–8 yr), subadult female (2–5 yr), adult male (8+ yr), and adult female (5+ yr).

Symptom	Year	Adult		Subadult		Juvenile		Yearling		Pup		Total
		M	F	M	F	M	F	M	F	M	F	
Acute	2004	1	2	1	0	1	0	0	1	1	0	7
	2005	1	2(2)	1(1)	1(1)	0	0	0	1(1)	0	0	6(5)
	2006	1	2	4(3)	1(1)	1	0	1	0	0	0	10(4)
	2007	6(2)	0	11(2)	1	2	0	0	0	1(1)	0	21(5)
	<b>Total</b>	<b>9(2)</b>	<b>6(2)</b>	<b>17(6)</b>	<b>3(2)</b>	<b>4</b>	<b>0</b>	<b>1</b>	<b>2(1)</b>	<b>2(1)</b>	<b>0</b>	<b>44(14)</b>
Chronic	2004	0	3	0	1	4	0	0	0	1	0	9
	2005	0	2	0	0	1	0	0	3	0	0	6
	2006	2(2)	2(2)	3(1)	4	2(2)	0	0	1	0	2	16(7)
	2007	1	1	2(1)	1	2	0	0	0	0	0	7(1)
	<b>Total</b>	<b>3(2)</b>	<b>8(2)</b>	<b>5(2)</b>	<b>6</b>	<b>9(2)</b>	<b>0</b>	<b>0</b>	<b>4</b>	<b>1</b>	<b>2</b>	<b>38(8)</b>
<i>(Other)</i>	2004	–	–	–	–	–	–	–	–	–	–	–
	2005	–	–	–	–	(5)	–	(1)	–	–	–	(6)
	2006	(1)	(1)	(3)	(1)	(16)	–	(1)	(2)	(4)	–	(29)
	2007	–	(1)	–	–	–	–	–	–	–	–	(1)
	<b>Total</b>	<b>(1)</b>	<b>(2)</b>	<b>(3)</b>	<b>(1)</b>	<b>(21)</b>	<b>–</b>	<b>(2)</b>	<b>(2)</b>	<b>(4)</b>	<b>–</b>	<b>(36)</b>

*P. pungens* were also present in the water. All the chronic cases had very low (ranging from 0.3 to 2.1  $\mu\text{g DA/g}$  with mean value of  $0.5 \pm 0.8 \mu\text{g DA/g}$ ) (50%, samples were mainly collected from 2006) or no detectable (50%) concentrations of DA in feces (Fig. 2E). Interestingly, the highest proportion (67%) of animals with detectable DA concentrations in feces was found in animals that stranded due to other causes (DA ranging from 0.2 to 40  $\mu\text{g DA/g}$ , with mean value of  $6.1 \pm 8.2 \mu\text{g DA/g}$ ), mostly young males (pup, yearling, juvenile and subadult) (Table 1).

*Table 2.* Summary of the regression results using Equations 1–6 as shown in Figure 4. All the estimated coefficients obtained from the regression analysis are shown, as well as standard deviation (SD), confidence interval for the regression analysis (CI), and  $r^2$  values.

Equations	Coefficients <sup>a</sup>					Statistics		
	<i>a</i>	<i>a</i> <sub>1</sub>	<i>a</i> <sub>2</sub>	<i>a</i> <sub>3</sub>	<i>b</i>	SD	95% CI	$r^2$
1	0.77				–1.89	2.20	0.69	0.49
2	0.17				–1.49	0.29	0.16	0.34
3	0.06				–0.39	1.49	0.47	0.04
4		–0.06	0.16		–0.10	0.77	0.24	0.20
5		0.09	–0.19		–0.03	0.64	0.20	0.32
6		–0.06	0.15	–0.02	–0.10	0.77	0.24	0.20

<sup>a</sup>The coefficients are defined in Equations 1–6 in Methods above.

## DISCUSSION

The presence of toxic cells in the water indicates the potential for toxin exposure via local pelagic food webs and the survival of intoxicated individuals may depend on the length of the bloom and cellular toxicity of the algal cells. These data indicated that toxic cells were detectable in the surface water during 2004 to 2007 and that cell numbers increased in abundance annually in the spring throughout the Bay. Overall cell abundance was, however, low. During 2004 to 2006 several delayed upwelling events occurred in Monterey Bay that resulted in generally low abundances of phytoplankton (Goericke *et al.* 2004, 2005; Peterson *et al.* 2006). Interestingly, during these years, rather than diatoms, dinoflagellates became the dominant phytoplankton in the Bay. However, in 2007 there was an increase in *Pseudo-nitzschia* presence and other diatom groups, compared to the previous 3 yr (Jester *et al.* 2009).

Overall for the study period, the highest particulate DA concentrations were detected in the spring of 2007 (March–May), which also corresponded with the highest number of sea lions stranding with signs of acute DA poisoning in the Bay and with highest DA concentrations in feces of individuals that exhibited acute signs of DA poisoning. Not all the acute cases tested for the presence of DA in feces in 2007 had detectable concentrations. This is not surprising due to the potential length of time between toxin exposure, stranding, being admitted to TMMC and sample collection. DA can decrease within individuals after 24 h of collection as the toxin is known to be poorly absorbed from the gastrointestinal tract and rapidly cleared from kidneys (Iverson *et al.* 1989, Suzuki and Heirlihy 1993, Truelove and Iverson 1994, Truelove *et al.* 1996). Thus, DA may not be detectable in feces in animals showing signs of acute DA toxicosis due to the varying doses to which they may have been exposed and to the varying lengths of times that may have occurred for each individual animal from exposure to stranding and sample collection.

All of the sea lions that stranded in the spring of 2007 were adult and subadult males. This finding, as previously reported by Bargu *et al.* (2010), is likely due to the fact that the normal onset of the breeding season for this species is in May, and thus the number of sea lions, especially adult and subadult males, may typically be higher in the Bay at this time just prior to their southward migration to breeding sites (Melin *et al.* 2000, Weise *et al.* 2006).

Sea lions also stranded with signs of acute DA poisoning throughout the Bay at other times when cell numbers of toxic *Pseudo-nitzschia* or particulate DA levels were low in the Bay, perhaps indicating alternate sources of DA for these animals. Toxin exposure may occur from different sources depending on where the blooms initiate and move, *i.e.*, the exposure or foraging location and stranding location may differ as sea lions may feed 30–60 km off the coast and they can travel up to 60–80 km in a day (Feldcamp *et al.* 1989, Melin *et al.* 2000, Weise *et al.* 2006).

In addition to stranded sea lions with acute DA poisoning signs, a neurological syndrome associated with chronic changes from sublethal exposure to the toxin also is now recognized (Goldstein *et al.* 2008). Although the number of chronic neurologic cases fluctuated each year, an overall increasing trend was observed, with the highest number stranding in 2006. This finding corroborates those by Goldstein *et al.* (2008) for a larger region of the California coast. Furthermore, in that study a time lag was found between strandings of subsequent chronic neurological cases following large acute events. As disease progression requires time to occur, the increased number of chronic cases in Monterey Bay in 2006 may be a consequence of the large bloom that

occurred in 2005 centered off the coast of San Luis Obispo and Santa Barbara counties that acutely affected a large number of sea lions (Goldstein *et al.* 2008, Schnetzer *et al.* 2007), and thus may explain, in part, the lack of association between sea lion strandings with chronic signs and toxic bloom presence. Behaviorally, animals with chronic neurologic sequellae have been shown to act significantly different from control animals at sea (Goldstein *et al.* 2008). DA associated brain lesions have been documented to affect their navigational abilities as affected animals traveled farther from shore, longer distances and stranded in varied locations (Goldstein *et al.* 2008, Thomas *et al.* 2010). Thus, this abnormal behavior, may in part, explain the increased presence of sea lions suffering from chronic effects of previous DA toxicity at varying times of the year in Monterey Bay, however, other factors such as food availability also affect sea lion movements and distribution (Feldcamp *et al.* 1989, Melin *et al.* 2000).

Clinical signs of low dose and repeated exposures in younger sea lion age classes are not as well defined as those seen in adult sea lions (Goldstein *et al.* 2008). Many of the sea lions categorized as "stranding without neurologic signs and stranded due to other causes" contained detectable DA concentrations in feces. The majority of these animals with detectable DA in feces (82%) were younger age classes (pup, yearling, and juvenile) (Table 2). In other species, increased doses are needed in young animals to observe the same clinical signs seen in adults, and adults have been shown to be more susceptible to the toxin than juveniles (Scallet *et al.* 1993, Truelove *et al.* 1996). The reasons for this are unknown, but the same may occur in sea lions. Alternatively, these animals may have ingested prey with a low DA dose that was detectable in feces by our methods, but low enough to not cause typical clinical signs, such as seizures, often associated with intoxication. Since the lowest dose needed to produce DA associated clinical signs in sea lions is unknown, both explanations may be plausible. Based on the domoic acid concentrations in feces in younger animals, some at the same levels measured in seizing adults, it appears that intoxication with domoic acid may go undiagnosed in some of these young animals. Additionally, dehydration associated with leptospirosis infection may decrease fecal and urine output (*e.g.*, Suzuki and Hierlihy 1993), thus when the animal does defecate DA may still be present in the sample, thus explaining the presence of DA in those samples for a longer period.

These data show that toxic *Pseudo-nitzschia* species are a part of the phytoplankton community in Monterey Bay but not always at bloom concentrations. As a result, sea lions are likely being exposed to varying levels of domoic acid in their prey throughout the year, often at sublethal doses that may contribute to a continued increase in the development of chronic neurologic cases. Additionally, the lack of recognized DA-associated clinical signs in young animals may lead to an underestimate of the number of affected sea lions. In conclusion, careful examination of stranding trends related to season, sex, and age class are crucial for detecting and quantifying the true potential impacts of domoic acid to sea lion populations, as well as in other marine mammal species.

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